**MONOAMINE OXIDASE IS A SOURCE OF OXIDATIVE STRESS IN OBESE PATIENTS WITH CHRONIC INFLAMMATION**

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MONOAMINE OXIDASE IS A SOURCE OF OXIDATIVE STRESS IN OBESE PATIENTS WITH CHRONIC INFLAMMATION

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Abstract

Obesity is an important preventable risk factor for morbidity and mortality from cardiometabolic disease. Oxidative stress (including in visceral adipose tissue) and chronic low-grade inflammation are the major underlying pathomechanisms. Monoamine oxidase (MAO) has recently emerged as an important source of cardiovascular oxidative stress. The present study was purported to evaluate the role of MAO as contributor to reactive oxygen species (ROS) production in white adipose tissue and vessels harvested from patients undergoing elective abdominal surgery. To this aim, visceral adipose tissue and mesenteric artery branches were isolated from obese patients with chronic inflammation and used for organ bath, ROS production, qRT-PCR and immune-histology (IH) studies. The human visceral adipose tissue and mesenteric artery branches contain mainly the MAO-A isoform, as shown by the qRT-PCR and IH experiments. A significant up-regulation of MAO-A, the impairment in vascular reactivity and increase in ROS production were found in obese vs non-obese patients. Incubation of the adipose tissue samples and vascular rings with the MAO-A inhibitor (clorgyline, 30 min) improved vascular reactivity and decreased ROS generation. In conclusion, MAO-A is the predominat isoform in human abdominal adipose and vascular tissues, is overexpressed in the setting of inflammation, and contribute to the endothelial dysfunction.

Key words: monoamine oxidases, obesity, inflammation, endothelial dysfunction
Introduction

Obesity is the most important modifiable risk factor for morbidity and mortality due to life-threatening cardiovascular diseases, type 2 diabetes and certain cancers. With the increased adoption of the sedentary and high-caloric intake lifestyles, particularly in developing/low-income countries, the prevalence of overweight and obesity continues to rise including among children (Fernandez-Sanchez et al. 2011; Hruby and Hu 2015). The expanded adipose tissue is responsible for the inflammatory environment (Pereira and Alvarez-Leite 2014) and high generation of reactive oxygen species (ROS), the two pathomechanisms of obesity-related complications (McMurray et al. 2016). Indeed, the low-grade chronic inflammation and oxidative stress at the level of adipose tissue (mainly visceral) are the major contributors to the occurrence of type 2 diabetes mellitus (DM) and accelerated atherosclerosis in the setting of obesity that currently affects 1/3 of people worldwide, hence the term “globesity” (Afshin et al. 2017).

Several intracellular sites have been identified as being responsible for ROS production, with the mitochondrial respiratory chain at the inner mitochondrial membrane (Muntean et al. 2016; Murphy 2009) and NADPH oxidases being particularly involved (Brandes and Schroder 2008).

In the past two decades, monoamine oxidases (MAOs) with 2 isoforms, A and B, at the outer mitochondrial membrane have emerged as important sources responsible for oxidative stress in the cardiovascular system in pathological conditions (Di Lisa et al. 2009; Kaludercic et al. 2014b). These flavin-dehydrogenases catalyze the oxidative deamination of neurotransmitters and biogenic amines (serotonin, dopamine and norepinephrine) with the constant generation of hydrogen peroxide, aldehydes and ammonia as deleterious by-products (Di Lisa et al. 2009; Kaludercic et al. 2014a).
MAO-A is classically considered the major isoform in the cardiovascular system that increases with ageing and is responsible for the related cardiac oxidative damage (Kaludercic et al. 2011) and also mediates the maladaptive evolution of ventricular hypertrophy towards heart failure (Kaludercic et al. 2014a), the endothelial dysfunction in the setting of hypertension (Sturza et al. 2013a; Sturza et al. 2013b), inflammation (Sturza et al. 2013a), chronic kidney disease (Utu et al. 2017) and diabetes (Duicu et al. 2016; Lighezan et al. 2016; Sturza et al. 2015b).

Scarce literature is available on MAOs contribution to oxidative stress in the adipose tissue. There are a couple of studies that reported an increased MAO activity in the white adipose tissue in obese mice (Tong et al. 1979) and dogs (Wanecq et al. 2006). As for humans, a pioneering study from the Parini's group identified high monoamine oxidase activities in abdominal and mammary human adipocytes and reported their role in the clearance of plasma norepinephrine (Pizzinat et al. 1999). More recently, MAO expression was reported to be increased during adipogenesis in human adipocytes (Bour et al. 2007).

However, no data are available concerning the role of these enzymes in the visceral adipose tissue in obesity associated with systemic inflammation. Thus, the aim of the present study was to investigate the MAO contribution to oxidative stress in adipose tissue and vascular samples harvested from patients with obesity and chronic inflammation.

**Material and methods**

Samples of omental adipose tissue and mesenteric arteries were obtained from consecutive patients undergoing abdominal surgery. Patients were randomized into two groups: (1) obese patients (OB) with an inflammatory status (n=10; 4 women, 6 men), and (2) non-obese (nonOB) without inflammation (n=10; 5 women, 5 men), respectively.
The University Committee for Research Ethics approved the study protocol (nr. 11/31.03.2017) and the informed consent was obtained from all patients prior to surgery, according to the World Medical Association Declaration of Helsinki.

**Immune-Histochemistry Studies**

Tissue expression of the MAO was quantified in frozen sections of human intra-abdominal adipose tissue and branches of mesenteric arteries using the MAO-A (Abcam, ab126751, 1:50) primary antibody and Alexa Fluor labeled secondary goat anti-rabbit antibody (Invitrogen, A32731, 1:200), respectively, as previously described (Sturza et al. 2015a; Sturza et al. 2015b; Sturza et al. 2018). Nuclear staining was obtained with DAPI (Santa Cruz, SC3598). The slides were examined using an Olympus Fluoview FV1000 confocal microscope (DAPI ex/em 405/461nm, Alexa Fluor ex/em 543/612nm). Images were analyzed with Icy, a free open source image analysis software, developed by the Quantitative Image Analysis Unit at Institute Pasteur (Paris) (de Chaumont et al. 2012).

**Oxidative Stress Assessment By Means of Ferrous Iron Xylenol Orange Oxidation Assay**

Hydrogen peroxide production was assessed in samples of visceral adipose tissue and branches of mesenteric arteries in the presence vs. absence of the MAO-A inhibitor, clorgyline (10 μM, 30 min incubation) using the Ferrous iron xylenol orange OXidation (FOX) assay (PeroxiDetect Kit, Sigma Aldrich) as previously described (Danila et al. 2017; Lighezan et al. 2016; Sturza et al. 2015b; Sturza et al. 2013a; Sturza et al. 2015c; Sturza et al. 2018; Utu et al. 2017). The principle of the assay is that peroxides oxidize Fe^{2+} to Fe^{3+} ions at acidic pH. The Fe^{3+} ion will form a colored adduct with xylenol orange, which is spectrophotometrically measured at 560 nm. Subsequently the production of H_2O_2 was calculated using the standard curve. The result is expressed in nmol H_2O_2/h/mg tissue.
Oxidative Stress Assessment By Means of Immune-Fluorescence

The total amount of ROS was determined using the DHE (dihydroethidium) probe according to a previously described technique (Duicu et al. 2016; Miller et al. 2008). Briefly, the samples were embedded in OCT and snap-frozen. The frozen fragments were cut in 8 μm thick cryosections and put on glass slides. After 3 washes with PBS, 5 minutes each, the cryosections were incubated in the dark with DHE for 30 minutes at room temperature. Excess DHE was removed by 3 additional washes with PBS. The slides were mounted with Vectashield (Vector Laboratories) and immediately analyzed in confocal microscope (Olympus Fluoview FV1000). Images were obtained using laser excitation at 488 nm. Image analysis was performed using Icy Bioimage Analysis software (de Chaumont et al. 2012).

Real Time Polymerase Chain Reaction (RT-PCR)

All tissues were homogenized using the Tissue Lyser (Qiagen). Total RNA was isolated (Total RNA Mini SI Isolation Spin-Kit, Applichem), measured using was verified using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and used for reverse transcription (Superscript III RT, Invitrogen). Quantitative RT-PCR (Biorad) was performed in adipose tissue and vascular samples. Primers against MAO isoforms were designed using sequence information from the NCBI database (5’→ 3’): human MAO-A forward: 5’-CTG ATC GAC TTG CTA AGC TAC-3’, human MAO-A reverse: 5’-ATG CAC TGG ATG TAA AGC TTC-3’. The housekeeping gene (EEF2, eukaryotic elongation factor 2) and its primers were as follow (5’→ 3’): EEF2 forward: GAC ATC ACC AAG GGT GTG CAG and EEF2 reverse: GCG GTC AGC ACA CTG GCA TA.

Vascular Reactivity Studies

The vascular samples (mesenteric artery branches) were mounted in the myograph (DMT) chambers filled 5 ml of Krebs solution (37°C) aerated with 95% O2-5% CO2 gas...
mixture (pH 7.4). The rings were stretched to an optimal tension of 1 g, allowed to equilibrate for 45 min, and further exposed to 80 mM KCl. The concentration of phenylephrine used for preconstriction was adjusted to obtain a preconstriction level of 80% of the contraction elicited by KCl (80 mM). Endothelium-dependent relaxation to cumulative concentrations of acetylcholine (Ach) and contractility to nitric oxide synthase (NOS) inhibitor L-NAME (10µM) were recorded in the presence vs. absence of irreversible MAO-A inhibitor, clorgyline (10 µM). Nitric oxide (NO) bioavailability was estimated from the constrictor response (%) to the classic NO synthases inhibitor, N-nitro-L-arginine (L-NAME, 10 µM) in vascular rings preconstricted with phenylephrine to 10% of the maximal KCl constriction.

Statistics

Data are presented as mean±SEM and were analyzed using a one-way ANOVA or student t-test when appropriate. Data analysis of the dose-effect response curves was performed using the ANOVA F-test (comparisons of bottom and top values, EC50 and the Hill slope). Values of p<0.05 were considered statistically significant.

Results

Characteristics of the study groups

The characteristics of patients included in the study are presented in Table 1. As shown, a mild inflammatory status was present in the obese group as compared to the non-obese one, as documented by the increased values of both CRP and ESR, the latter being the hallmark of a chronic inflammatory status. Also, a significant lower value of HDL was found in the obese patients.

MAO Is Expressed In The Visceral Adipose Tissue And Mesenteric Artery Branches
Limited information is available regarding the MAO expression in human visceral adipose tissue and mesenteric vessels. For this reason we evaluated the expression of MAO isoforms in adipose tissue samples and arterial fragments isolated from patients subjected to elective abdominal surgery by means of IH. The results showed that both MAO isoforms were present with predominance of the MAO-A (Fig. 1A). MAO was diffusely distributed in the adipose tissue whereas in the vascular wall we noticed an increased expression in the intima and the adventitia (Fig. 1A).

*Obesity And Chronic Inflammation Are Associated With Increased MAO-A Expression In Both Adipose Tissue And Vasculature*

Increased oxidative stress in the adipose tissue and vasculature is the hallmark of overweight and obese patients but the sources of ROS are partially elucidated. While the classical sources still remain NADPH oxidases and the mitochondrial respiratory chain, we report here that MAO-A isoform contributes to the adipose tissue and vascular oxidative stress, being up-regulated in samples isolated from obese patients with chronic inflammation, as revealed by the mRNA gene expression (Fig. 1B).

*MAO-A Is A Source Of ROS in Adipose Tissue And Vasculature When Obesity Coexists With Inflammation*

As MAO-A was up-regulated in the adipose tissue and vascular preparations from obese patients we further assessed the ROS production by IH and FOX assay after incubating the samples with the MAO-A inhibitor, clorgyline (10 μM, 30 min). *Ex vivo* incubation with clorgyline was able to reduce the signal related to oxidative stress as revealed by DHE staining (Fig. 2A) and rate of hydrogen peroxide production measured by FOX assay (Fig. 2B).
MAO Inhibition Improves Vascular Reactivity of Mesenteric Branches Isolated From Obese Patients With Inflammation

Since we noticed an upregulation of MAO-A together with an increased amount of ROS in the vessels we thought to determine whether MAO inhibition is also able to modulate the reactivity of the vascular preparations in organ bath studies. Vascular contractility in samples obtained from obese patients with inflammation was significantly increased in response to cumulative doses of phenylephrine whereas the endothelium-dependent relaxation was significantly attenuated. Incubation with the irreversible MAO-A inhibitor restored both the contractile and relaxation responses. Also, contractility to L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride, 10 μM), the classic NO synthases inhibitor, was significantly increased in samples from obese patients; this response was reduced after clorgyline, a finding that strongly suggests the involvement of NO in the vascular protective effect of MAO inhibition.

Discussions

The main finding of this pilot study is that MAO-A isoform contributes to the adipose tissue and vascular oxidative stress in the setting of obesity and systemic inflammation. These observations are particularly important since a persistent, dysfunctional low-grade inflammatory status is the hallmark not only of obesity but also of all non-communicable chronic diseases of the 21st century (Bennett et al. 2018). The obesity-driven changes of the adipose tissue microenvironment are responsible for the chronic systemic inflammation via the upregulation of the pro-inflammatory cytokines and the downregulation of the anti-inflammatory ones, as recently reviewed in (Fuster et al. 2016).
Also, the increased oxidative stress in the setting of obesity has been systematically investigated, in particular the role dysfunctional complexes of the electron transport chain (McMurray et al. 2016). Here we unequivocally demonstrated that MAO is an important contributor to the oxidative stress in obese patients with chronic inflammation. Indeed, MAO inhibition was able to significantly reduce the amount of H$_2$O$_2$ in the white adipose tissue and vascular samples from obese patients. Of note, in a previous study we firstly noticed the presence of MAO in the perivascular adipose tissue of coronary arteries isolated from patients with coronary artery disease (Lighezan et al. 2016) - an observation that prompted this study. In our hands, *ex vivo* MAO inhibition elicited beneficial effects in several studies performed in human vascular samples, such as internal mammary arteries isolated from patients subjected to coronary by-pass surgery (Lighezan et al. 2016) and in collaterals of brachial artery in patients with chronic kidney disease with indication of hemodialysis (Utu et al. 2017).

Interestingly, irreversible MAO-A inhibition with clorgyline elicited a significant reduction of oxidative stress only in adipose tissue samples harvested from the obese group as revealed by the DHE staining and FOX assay, respectively. However, in organ bath experiments, clorgyline was able to reduce contractility in all the vascular rings, regardless the study group. This observation is suggestive for both tissue specificity and pleiotropic effects of MAO inhibitors (behind MAO inhibition). In an earlier study we investigated whether MAO inhibitors act as ROS scavengers, a hypothesis that was not confirmed (Sturza et al. 2013a). However, since clorgyline was able to reduce the contractility to L-NAME, we speculate that its beneficial effect is mediated via an interaction with the NO-dependent-pathway. In this respect, we have previously observed in HUVECs that MAO directly interfere with NO signaling pathway and limit the accumulation of cGMP (Sturza et al. 2013a).
The presence of MAO-A has been documented together with the one of semicarbazide sensitive amine oxidase (SSAO) in the mesenteric perivascular adipose tissue from rats (Ayala-Lopez et al. 2017). Interestingly, these authors reported that individual inhibition of either enzyme did not alter the contractility to norepineprine; however, inhibition of both MAO and SSAO increased the effect of norepinephrine on mesenteric arteries with peripheral adipose tissue. We acknowledge as limitations of our study the fact that we did not assess the enzymatic activity and protein expression or putative crosstalk with other amine oxidases. Also, the level of free fatty acids which may directly interfere (when increased) with the vascular reactivity via a direct membrane effect was not measured.

The constant interest in studying ROS sources in the cardiovascular system stems from the fact that cardiovascular diseases and their comorbidities (obesity, metabolic syndrome and diabetes mellitus) remain the leading causes of morbidity and mortality worldwide, despite a huge and constantly expanding therapeutic armamentarium. All of these pathologies have common pathogenesis that include the triple association of endothelial dysfunction, "low-grade" inflammation and oxidative stress, all cumulatively contributing to disease progression and complications. Nevertheless, whether the increased MAO expression in the visceral adipose tissue is a cause or a consequence of the prolonged low-grade inflammatory status remains an open question.

MAO inhibitors have been systematically studied starting from the mid-past century for their therapeutic efficacy in neurodegenerative and psychiatric illnesses (including depression) via complex mechanisms that include the mitigation of the oxidative stress (Bortolato et al. 2008; Song et al. 2013). Therefore, in line with the recently reported association between a higher body mass index (BMI) and depression, particularly in women (Tyrrell et al. 2018) the study of the new generations of reversible MAO inhibitors as potential candidates for drug repurposing in the treatment of cardiometabolic diseases
represents a feasible alternative. A starting point could be represented by the assessment of the effects of MAO inhibition on the systemic inflammatory status in the setting of chronic non-communicable diseases.

**Conclusions**

In conclusion, monoamine oxidases are expressed in human visceral adipose tissue and branches of mesenteric artery and the MAO-A isoform is upregulated in the setting of obesity associated with chronic inflammation. *In vitro* inhibition of MAO-A significantly reduced the amount of oxidative stress in both adipose tissue and arteries; in the latter an improved vascular reactivity (reduced contractility and increased endothelium-dependent relaxation) was also found. These data also suggest that MAO inhibitors are promising candidate compounds for drug repositioning.

**Acknowledgements**

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**Conflicts of interest**

The authors declare that there is no conflict of interest regarding the publication of this paper.
References


**Figure captions**

**Figure 1.** MAO is expressed in human adipose tissue and vasculature and is up-regulated in setting of obesity. (A) MAO expression in human intra-abdominal adipose tissue and mesenteric artery branches in Immune-fluorescence: Green - anti-MAO-A antibody, blue - DAPI. (B) MAO-A expression (fold change) in visceral adipose tissue and mesenteric artery branches from obese (OB) and non-obese (nonOB) patients – qPCR, *p<0.05.

**Figure 2.** MAO is a source of oxidative stress is human adipose tissue and vasculature. The effect of MAO-A inhibitor clorgyline (Clorg, 10 μM) on oxidative stress in (A) adipose tissue and (B) mesenteric artery branches samples harvested from obese (OB) and non-obese (nonOB) patients – DHE stain. (C) The effect of MAO-A inhibitor clorgyline (Clorg, 10 μM) on hydrogen peroxide production in adipose tissue and mesenteric artery branches from obese (OB) and non-obese (nonOB) patients – FOX assay. *p<0.05.

**Figure 3.** MAO-A inhibition improves vascular reactivity in human mesenteric artery branches. (A) Phenylephrine-induced contractions, (B) Acetylcholine-induced endothelium-dependent relaxation (C) Contraction to L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride, 10μM), in human mesenteric arteries branches obtained from obese (OB) and non-obese (nonOB) patients, incubated or not with MAO-A inhibitor, clorgyline (Clorg, 10 μM, 30 min). *p<0.05 OB vs. nonOB, #p<0.05 OB vs. OB+Clorg.
Table 1. Characteristics of the study groups.

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Data are means ± S.E.M. and differences are highlighted in bold.
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